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Differentiation of Mammary Stem Cells *In Vivo* and *In Vitro*

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The fully differentiated cells of the rat mammary parenchyma, the ductal epithelial, alveolar, and myoepithelial cells, are distinguished by their ultrastructure and by their accumulation of immunocytochemically detectable marker proteins. The different cell types probably develop from primative ductal structures called terminal end buds, which are present in the developing rat mammary glands, and these structures contain relatively undifferentiated cells.

Clonal epithelial stem cell lines, obtained from normal rat mammary glands or benign mammary tumors, differentiate under appropriate conditions along a pathway to droplet-cell/doming cultures of primative alveolarlike cells. Under different culture conditions, the epithelial stem cells differentiate along a separate pathway to myoepitheliallike cells. They accumulate some of the specific marker proteins of myoepithelial cells *in vivo*, including type IV collagen, laminin, and Thy-1 antigen. In addition, these myoepitheliallike cells in culture contain an abundance of a potential calcium-binding protein, p9Ka, which also occurs in myoepithelial cells of histological sections from mammary glands.

The accumulation of type IV collagen, laminin, Thy-1, and p9Ka occurs asynchronously along the pathway to the myoepitheliallike cells *in vitro*. Furthermore, the steady-state levels of these different marker proteins arise by alterations in the controls at the transcriptional, the posttranscriptional processing, and the translational stages of their production. These results suggest a stepwise control of synthesis of myoepithelial cell marker proteins, and in the case of p9Ka and Thy-1 antigen, this altered control may arise through their possession of novel transcriptional promoters.

Differentiation of the Mammary Gland and Its Tumors *In Vivo*

The mammary gland of the adult female rat consists of a system of branching ducts terminating in alveoli and embedded in a fatty stroma (*1*). The mammary ducts are composed of one or more layers of cuboidal, epithelial cells, some of which border a lumen that is continuous throughout the ductal system. The epithelial cells are surrounded by a layer of elongated, myoepithelial cells (*2,3*). These two fully differentiated cell types have been distinguished in the past by their characteristic ultrastructural morphologies. The ductal epithelial cells possess apical microvilli and specialized junctional complexes with associated desmosomes, whereas the myoepithelial cells possess smooth musclelike myofilaments with pinocytotic vesicles and basement membrane on their basal surfaces. A third functionally differentiated cell type, the secretory cell, is found in the mammary alveoli. This cell type is characterized by its ultrastructure and, during lactation, by the synthesis and secretion of milk products (*4,5*).

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More recently, immunocytochemical stains have been used to distinguish the ductal epithelial, myoepithelial, and alveolar cell types. The epithelial cells are stained, for example, by antisera to milk fat globule membranes (*6*) and keratin monoclonal antibody LE61 (*7*). The myoepithelial cells are normally stained by antisera to vimentin, actin, myosin (*6,8*), keratin monoclonal antibody LP34 (*7,9*), and by the lectins *Griffonia simplicifolia*-1 (GS-1) and pokeweed mitogen (*10*). The basement membrane, which is closely associated with and possibly synthesized at least in part by the myoepithelial cells, stains with antisera to laminin, type IV collagen, and Thy-1 antigen (*6,8,11,12*). The secretory cells are characterized by being stained with peanut lectin (*13*) and, during lactation, with antisera to casein (*14*).

In the rat, the development of the mammary parenchyma takes place predominantly after birth but before puberty (*15*) by the lengthening and branching of primative ducts within the mammary fat pad. During this period of growth, the ducts terminate in globular structures called terminal end buds (TEBs), which contain most of the dividing parenchymal cells (*16*). The number of TEBs reaches a maximum in rats about 20 days old (*16*), and thereafter the number rapidly declines as the TEBs differentiate to terminal ducts and alveolar buds (*17*). The alveolar buds are the direct precursors of the secretory alveoli. The TEBs consist of a heterogeneous collection

of cells that show a gradation in ultrastructural and immunocytochemical-staining characteristics toward the epithelial cells within the cortex of the TEB on the one hand, and to the myoepithelial cells of the subtending duct on the other hand (*18,19*). Although it is probable that all the alveolar cells may arise from differentiation events within the TEBs and alveolar buds, it is not entirely clear whether all the myoepithelial cells arise in this way.

Myoepithelial cells are absent from the developing ducts of the mammary glands of young rodents until about 7 days after birth (*6,20*). Dissection of part of the rodent mammary gland and transplantation to other suitable sites in syngeneic animals results in the generation of fully developed mammary glands at the sites of implantation. These implanted glands will secrete milk products in isologous, pregnant hosts (*21,22*), suggesting a reversible state between ductal and alveolar cells. Bud-free ducts dissected from rat mammary glands can regenerate the entire mammary parenchyma including the TEBs (*23*) and casein-producing alveoli in appropriate hosts (*24*). These facts raise the possibility that fully differentiated myoepithelial cells may also arise from cells present within the bud-free ducts.

The susceptibility of the rat mammary gland to chemical carcinogenesis correlates with the presence in the gland of TEBs and terminal ducts (*25*). The tumors induced by dimethylbenzanthracene (DMBA) (*26*) or nitrosomethyl urea (NMU) (*27*) are predominantly cytologically benign in the authors' experience (*28*). These relatively benign tumors contain areas of epithelial and elongated, myoepitheliallike cells in ductlike arrangements (*29*). However, many of the elongated, myoepitheliallike cells possess a more undifferentiated appearance than the myoepithelial cells of mature mammary ducts (*30*). Hormonal stimulation of the host leads to production of a small proportion of alveolarlike cells that can synthesize casein (*31*). However, the amount of casein and casein mRNA produced by these cells is only 1 to 5% of that produced by the alveolar cells of the lactating mammary glands in normal rats, when animals bearing the tumors are subsequently mated (*14,31,32*).

Morphological Differentiation of Cultured Normal and Tumorous Mammary Stem Cells

Limited digestion of carcinogen-induced benign mammary tumors, or normal mammary glands, yields organoids that will subsequently adhere to the surface of a tissue culture vessel, providing that their basement membrane has not been destroyed. The stromal cells adhere to the substratum after about 2 hr, and the epithelial fraction adhers after 12 to 24 hr (*33,34*). After several days in culture, epithelial cells grow from the organoids and single epithelial cells can be cloned (*35*). Such single-cell-cloned epithelial cell lines have been obtained from the normal mammary glands of 7-day-old inbred, Furth-Wistar rats (*36*), from DMBA-induced tumors of outbred Sprague-Dawley rats (*35*) or inbred, Furth-Wistar rats (*37*), and from an NMU-induced rat mammary tumor (*38*). All the cell lines derived from the normal mammary glands or benign tumors behave in a similar way. Thus, the work on the benign tumor-derived epithelial cell line Rama 25 is described in detail.

Sequential Changes Associated with Differentiation to Alveolarlike Cells *In Vitro*

Rama 25 cells grow on collagen gels and form branched, ductlike structures with a correctly organized basement membrane (*39*). Such structures are reminiscent of the immature ducts found in neonatal rat mammary glands. That the Rama 25 cell line can form such ductlike structures suggests that it may represent a mammary stem cell. However, Rama 25 cells are more conveniently cultured on a plastic substratum on which they grow with a cuboidal, epithelial morphology. When such cultures, growing in the presence of hydrocortisone and insulin, become densely packed, small, dark, polygonal cells are formed (*35*) that contain vacuoles or droplets at their peripheries (droplet cells). These cells form hemispherical blisters or domes that are thought to arise from the unidirectional pumping action of the ouabain-sensitive sodium/potassium ATPase (*40*).

The pathway of morphological differentiation *in vitro* to the droplet/doming cultures takes place through a linear sequence of morphological stages in the order cuboidal, grey, dark, dark-droplet, and doming cells (*41*). The overall process can be accelerated by the addition of the erythroleukemic cell-differentiation inducer, dimethyl sulfoxide (*35,42*), or by retinoic acid (*43*) in the presence of the mammotrophic hormones prolactin, estrogen, hydrocortisone, and insulin. The doming cultures can synthesize rat casein as detected by radioimmunoassay and this casein has a peptide map identical to that of rat β-casein (*44*). However, the amount of casein produced by Rama 25 cells is 50- to 100-fold less than that synthesized by lactating rat mammary glands or cultured explants (*44*). This low level of casein production by the doming Rama 25 cells may reflect the fact that the Rama 25 cell line was originally derived from a mammary tumor.

The discrete morphological stages in the formation of the casein-secreting doming cultures *in vitro* are characterized by parallel changes in a small number of specific polypeptides (*45*); however, the identity and occurrence *in vivo* of many of these stage-specific polypeptides has not yet been determined. Droplet, doming cultures of Rama 25 cells also can bind peanut lectin, but only after they have been treated with neuraminidase. In this respect the cultures resemble cells of the alveolar buds of the developing mammary gland, which also require prior neuraminidase treatment before peanut lectin will bind. The better developed alveolar cells, however, can bind peanut lectin extensively without their prior treatment with neuraminidase (*13*). This result suggests that Rama 25 cells probably resemble cells of the early developmental stages in the rat mammary gland, and this may further account

for the lack of expression of the fully differentiated phenotype, for example, the abundant synthesis of casein.

Changes Associated with Differentiation to Myoepitheliallike Cells *In Vitro*

Although Rama 25 cells have been single-cell cloned three times, confluent cultures at high passage number yield ridges of elongated cells (*46*), and subconfluent cultures yield 1 to 3% of clones of cells with an elongated morphology (*35*). The formation of similar morphological forms occurs also in cultures of mouse mammary tumors (*47–49*) and in cultures of normal rat mammary glands (*36*). From a comparison of the ultrastructure and immunocytochemical staining characteristics of histological sections of rat mammary glands (*39,46*) and of their primary cultures (*50*), the elongated cells derived from cultures of cell line Rama 25 are thought to be related to myoepithelial cells.

In primary cultures, myoepithelial cells stain with antisera to vimentin (*50*), Thy-1 (*24*), laminin, type IV collagen (*50,51*), and the lectins GS-1 and pokeweed mitogen (*10*), similar to the staining of myoepithelial cells and basement membranes *in vivo*. However, there are some differences; thus, antisera to actin and myosin stain only some of the elongated, myoepitheliallike cells in primary culture (*50*). In a similar fashion to the primary cultures, the elongated, myoepitheliallike converts from the cloned mammary epithelial cell lines always express vimentin, laminin, type IV collagen, Thy-1 (*12,46,52–55*), and receptors for the lectins GS-1 and pokeweed mitogen (*10*). But, as with primary cultures, some elongated cell clones stain well with actin and myosin; others (e.g., Rama 29 cells) stain poorly (*56*). In general, cells of a more mature myoepithelial phenotype have been derived from the epithelial cells of normal rat mammary glands, for example, cell lines Rama 704E (*36*) and Rama 401 (*54*), than from the epithelial cells of mammary tumors, for example, Rama 29 (*35*) and Rama 37E5 (*37*). This result is consistent with the finding that the better differentiated myoepithelial cells occur in normal mammary glands rather than in their tumors. Moreover, subcloning of the better-differentiated myoepitheliallike cell lines *in vitro* can also lead to a further loss of the microfilamental systems and to a less differentiated cellular phenotype (*57*). Thus, based on these results, the majority of the more elongated cells *in vitro* are classified as myoepitheliallike rather than as mature myoepithelial cells (*58*).

Polypeptide Changes Associated with the Differentiation to Myoepitheliallike Cells *In Vitro*

The conversion in culture of the cuboidal, epithelial cells to elongated myoepitheliallike cells is accompanied by changes in a small number of specific polypeptides. Essentially the same changes are also found for the conversion of epithelial cells derived from normal glands or from benign tumors to the elongated myoepitheliallike cells (*55*). The pattern of acidic polypeptides from the elongated, myoepitheliallike cell line, Rama 29, and that from the parental cuboidal, epithelial cell line, Rama 25, are shown in Figure 1. The patterns are very similar, only 6% of the total number of polypeptides resolved show any quantitative or qualitative changes (*59*) and of these only nine polypeptides show major changes. Three polypeptides show a marked decrease in the elongated cells, whereas six are more abundant. Polypeptides 3–9 are components of the intermediate filaments within the cells (*59*). The same differences are found irrespective of whether the proteins are located by staining or by radioactive labeling. However, both systems detect only the most abundant proteins in a cell; the other proteinaceous markers of the myoepitheliallike cells, Thy-1, laminin, Type IV collagen, the receptors for GS-1 and pokeweed mitogen, are of much lower abundance and are therefore not readily detected using this technique. However, a novel polypeptide of molecular weight 9000 (Number 1 in Fig. 1), termed p9Ka, is present in abundance in the myoepitheliallike cells but is barely detectable in the parental, cuboidal epithelial cells.

The increase in synthesis of p9Ka by the elongated, myoepitheliallike cells reflects the presence in these cells of an increased level of translatable p9Ka mRNA when

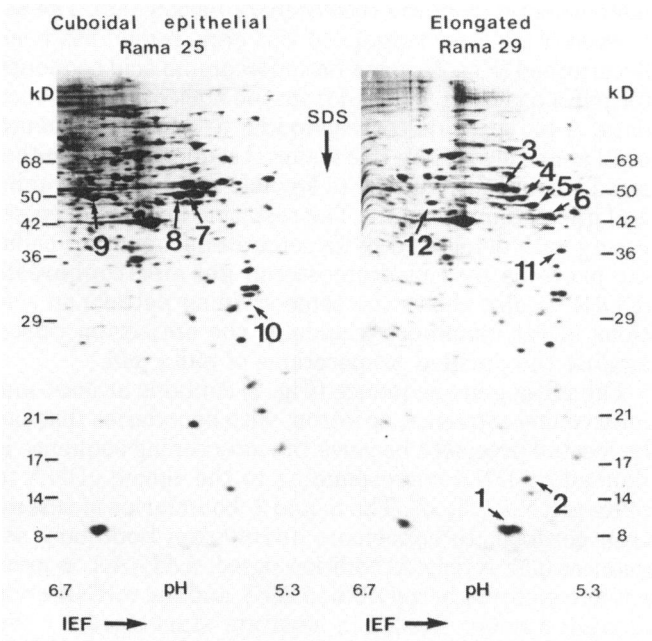

FIGURE 1. The pattern of polypeptides of cuboidal epithelial and elongated, myoepitheliallike cell lines following two-dimensional gel electrophoresis. Extracts of cuboidal epithelial cells, Rama 25 (left panel) or elongated, myoepitheliallike cells, Rama 29 (right panel) radioactively labeled overnight with [^{35}S]methionine, were subjected to isoelectric focusing in the first dimension, and SDS gel electrophoresis in the second dimension. The autoradiographs of the dried-down gels are shown. The large numbers superimposed on the gels refer to polypeptides that show major quantitative or qualitative variation between the two cell lines. Polypeptides 7, 8, and 9 show a marked decrease in the elongated cells relative to the cuboidal cells; polypeptides 1–6 are more abundant in the enlongated cells than in the cuboidal cells.

compared with the parent cuboidal epithelial cells. The protein p9Ka has been purified 63-fold from the elongated, myoepitheliallike cells using a combination of acid extraction and high-performance liquid chromatography. The resulting preparation of p9Ka shows only a trace of contaminating polypeptides on SDS polyacrylamide gels, and appears homogeneous on two-dimensional polyacrylamide gels when they are stained by Coomassie blue. Although p9Ka is not a particularly antigenic protein, a mouse antiserum has been raised to the relatively small amounts of purified preparations of p9Ka that have been available so far. This mouse antiserum binds to myoepithelial cells and smooth muscle in histological sections of rat mammary glands (*60*).

A Polypeptide Marker for Myoepitheliallike Cells is Related to a Class of Calcium-Binding Proteins

A cloned cDNA corresponding to p9Ka mRNA has been obtained from a cDNA library constructed with mRNA from the elongated, myoepitheliallike cell line, Rama 29 (*61*). It is complementary to 400 bases at the 3′ end of the p9Ka mRNA. This cloned cDNA has been used to screen a genomic library containing fragments of rat liver DNA. An 18-kilobase fragment of rat genomic DNA that contains the p9Ka gene and its flanking regions has been isolated from the recombinant library (*62*). The sequence of 2340 nucleotides of this gene region has been determined (Fig. 2), and a potential amino acid sequence for p9Ka has been deduced from the nucleotide sequence data. A polypeptide corresponding to a linear sequence of 12 amino acids from this deduced sequence was synthesized, chemically coupled to keyhole limpet hemocyanin, and injected into rabbits. The resulting antiserum cross-reacts with denatured p9Ka separated from other cellular proteins by two-dimensional gel electrophoresis (*63,64*). It also shows the same staining pattern on sections of rat mammary glands as the antiserum raised against the purified preparations of p9Ka (*60*).

The p9Ka gene sequence (Fig. 2) contains at least one intervening sequence, or intron, with boundaries that can be located precisely, because the intervening sequence is flanked by DNA corresponding to the cloned cDNA to p9Ka mRNA (Fig. 2). The 5′ and 3′ boundaries of this intron conform to concensus intron/exon boundary sequences (*65*). A poly(A) addition signal, AATAAA, is present at bases numbered 2176 to 2181, and the mRNA ends at base number 2194. Six separate start sites for the mRNA have been located to a small region of the DNA between bases numbered 834 and 884. The size of the p9Ka mRNA is thus 629 to 680 nucleotides and is consistent with the actual size of 700 to 780 nucleotides including the poly(A) tail, when measured following agarose gel electrophoresis (*61*). When the same nucleotide sequence is translated in all three possible reading frames, it contains only one open reading frame of sufficient length to encode a polypeptide of 9000 molecular weight. This reading frame contains a potential coding region of 100 amino

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        10         20         30         40         50         60         70         80         90
GGATCCAGATGAGAGATTCTGGTACGGAGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTATTGCACAAGA

       100        110        120        130        140        150        160        170        180
ATGAAAACTGAAAAACAAGCAGTATATAAATGGCTCCCGGAGATTCTGAGATGCTGAGGCTTGCTTGTATGTTGCTATAGTGTATGTTGG

       190        200        210        220        230        240        250        260        270
TGCTTGGGAGCCACTGTCATGGATAGGTATGTTGCTGGGTCATCCAAGCCAGTGTGTGGACACTCAGGTACAGGAAGCAAAGTGAAGGCA

       280        290        300        310        320        330        340        350        360
TCAGCAGGCATTTTTGTTTTACGATGTTTAAATTACACTTATTTTATTTGTGTGTACGAGTGTATGGGTGGGGATGGGGCAAATGCCAAG

       370        380        390        400        410        420        430        440        450
GGGCACTTCTTGTGAGAGTCAATCTGTTCCTTCTAGCATGTGGGCTCTGGAGATCAAACTCAGGTCATTGAGCTTGGTGGCAAGCACCTC

       460        470        480        490        500        510        520        530        540
TACCTACTGAGCCACCTGTTCAACACCCACCTGTAGGCATTTGTGTTCATAGTAGTTCATAGCCCTATGAACATATAGCACCTAGGCCAA

       550        560        570        580        590        600        610        620        630
GAGAGCCTGGCTTCCCCACCCCCTCCCCTTGTACCCCAACCTCTGCCACTTCATCTCACTCCTACTAGGCAGCTGGGTTTTTTCCCTCAC

       640        650        660        670        680        690        700        710        720
TGTAGGCCCCTGGGCAGGCAGCCAGCAGCCGCGCCCAACGCTGGGAGGGAGAAGAATGGGTCAGAGGCTGGAGCTTGTGGTTGAGTTGGG

       730        740        750        760        770        780        790        800        810
GAGTGAGTAAGCTGAGTGAGGGATGGAAAACTGCTGTTGTTGAGGCCAGGCCTGGGGGGGAGGCACAGAAGGCTGCTGGCATGAATTTCT

       820        830        840        850        860        870        880        890        900
AGAGTTTGAGTGGTAAGTTTTGCAAGTTTCAGAGCTTGAAGCACATATGAGCTTCTTGCCATCAGTGGGTACCACTCCTCTGATCTCCCT

       910        920        930        940        950        960        970        980        990
GGGAGTGAGGTCGGTCTCTGGAAGTGCTCTTAGAGAGTAGGTTGGAGTAGAGCACTAAAAACGGGGACAGACTGAGTGTGACTTGAGTGA

      1000       1010       1020       1030       1040       1050       1060       1070       1080
TGCCTAGCAACATATATCCAGCTCTCAACACACTGTTGGTGTGGGTTGGAGAAGGCTACTTTTGTGTCTCCTGCCCCTAGGTCTCAACGG

      1090       1100       1110       1120       1130       1140
TCACCATG GCG AGA CCC TTG GAG GAG GCC CTG GAT GTA ATA GTG TCC ACC TTC CAC AAA TAC TCA GGC
     Met Ala Arg Pro Leu Glu Glu Ala Leu Asp Val Ile Val Ser Thr Phe His Lys Tyr Ser Gly

          1160       1170       1180       1190       1200       1210
AAC GAG GGT GAC AAG TTC AAG CTG AAC AAG ACA GAG CTC AAG GAG CTA CTG ACC AGG GAG CTG CCT AGC
Asn Glu Gly Asp Lys Phe Lys Leu Asn Lys Thr Glu Leu Lys Glu Leu Leu Thr Arg Glu Leu Pro Ser

          1230       1240       1250       1260       1270       1280       1290       1300
TTC CTG GGG gtgagtggatcctgtctgtgtattgcatatgtgatgcatccccaggaggaggctggggctggagatatctatctatct
Phe Leu Gly

  1310       1320       1330       1340       1350       1360       1370       1380       1390
atctatctatctatctatctatctatctatctatctatctatctatctatatctatatcatctatctatatctatctatctatcta

  1400       1410       1420       1430       1440       1450       1460       1470       1480
catatatatatatatatatatatatatatatatatatatatatatatctctccctactcctggcgcttggtatggaaccacaatga

  1490       1500       1510       1520       1530       1540       1550       1560       1570
accatctacttcacaccagccccccgttgagacaaggcttagaatgaagttaactgaagtggcacaggaaaaccacattaggtagtcagt

  1580       1590       1600       1610       1620       1630       1640       1650       1660
gtctgaaagcacagcctagatcaggacagtctttccggtgatgtgcaacagaaatcgagtttctgcttgtgaagacatgattgtggaggc

  1670       1680       1690       1700       1710       1720       1730       1740       1750
acacaaatgcctgcagatcttcccctcaatgacaccttatcttagttaacacctccttgtcatgacagttacctatagacatagttaaaa

  1760       1770       1780       1790       1800       1810       1820       1830       1840
caagcgtggggaagatgtggtcacatcctttcccagctagcccatgtgctcatctcacagttgagccctgaggctagcacggtgtctgca

  1850       1860       1870       1880       1890       1900       1910       1920
agccttcctgagctcctggctggaggtggcgtctaactgtacctcttctacctccag AGA AGG ACA GAC GAA GCT GCA TTC
                                                           Arg Arg Thr Asp Glu Ala Ala Phe

  1930       1940       1950       1960       1970       1980       1990
CAG AAG CTG ATG AAC AAC TTG GAC AGC AAC AGG GAC AAT GAA GTT GAC TTC CAG GAG TAC TGT GTC TTC
Gln Lys Leu Met Asn Asn Leu Asp Ser Asn Arg Asp Asn Glu Val Asp Phe Gln Glu Tyr Cys Val Phe

    2000       2010       2020       2030       2040       2050       2060
CTG TCC TGC ATT GCC ATG ATG TGC AAT GAA TTC TTT GAG GGC TGC CCA GAT AAG GAG CCC CGG AAG AAG
Leu Ser Cys Ile Ala Met Met Cys Asn Glu Phe Phe Glu Gly Cys Pro Asp Lys Glu Pro Arg Lys Lys

      2070       2080       2090       2100       2110       2120       2130       2140
TGA AGA CTC CTC AGA TGAAGTGTTGGGCCAGTGGGGGAATCTTCCATGTTGGCTGTGAGCATAGTGCCTTACTCTGGCTTCTTCA
End                 End

          2160       2170       2180       2190       2200       2210       2220       2230
TACATGTGCACAGTGCTGAGCAAGTTTAATAAAGAGTTTTGAAACTatgtctgagagactggagattgtgggtgggtgggtgggctgagg

          2250       2260       2270       2280       2290       2300       2310       2320
gagggtggtggcagagatggctggaagttgacctggagctttgtggggccaactagaaaaggttgggggaggggtgagtgactatctgagt

       2340
catacagtgtat
```

FIGURE 2. The nucleotide sequence of the p9Ka gene. The sequence of the strand of DNA corresponding to the mRNA of p9Ka is shown. The single intron is shown in lower case letters from nucleotide numbered 1227 to nucleotide numbered 1901. The potential p9Ka coding region is shown with the corresponding amino acid residues beneath. The sequence corresponding to the p9Ka cDNA is underlined. The line above the sequence indicates the single EcoRI site.

acids, excluding the initiating methionine residue and ends with two termination codons.

The potential amino acid sequence for p9Ka is shown in Figure 3. A search of computerized data bases of protein and nucleic acid sequences shows limited homology (*66*) of this potential p9Ka amino acid sequence to both the α (43%) and β (43%) chains of bovine S-100 protein (*67,68*) and to rat S-100 protein (42%) (*69*). This homology is also observed in the corresponding nucleic acid sequences. A cloned cDNA corresponding to a portion of rat S-100 pro-

FIGURE 3. The potential amino acid sequence of p9Ka and its relationship to S-100 protein and bovine intestinal calcium-binding protein. Amino acids are shown using the three letter code and are arranged according to the maximum homology using the algorithm of Dayhoff (*66*). Amino acids in the sequence of S-100 protein and of bovine intestinal calcium-binding protein are shown in bold type. The stars between the sequences show preferred side chains for the EF-hand structure as indicated by Szebenyi et al. (72). Asterisk (*) indicates an amino acid with a hydrophobic side chain; double asterisks (**) indicate an amino acid with an oxygen-containing side chain; (G) indicates glycine. The helix, loop, helix arrangements of the calcium-binding sites are indicated by underscoring.

tein mRNA (*69*) has an overall homology of 66% with the corresponding region of the p9Ka gene.

The protein p9Ka also shows a weak homology (34%) with the bovine vitamin D-dependent, intestinal calcium-binding protein (Fig. 3) (*70*). This protein has two potential calcium-binding sites, one of which conforms to the EF-hand structure of known calcium-binding proteins (*71,72*). When only those amino acid residues thought to be involved in calcium binding by the two sites of bovine intestinal protein (*72*) are compared with those in similar positions in p9Ka, 11 out of 14 of these residues are identical in the two polypeptides. The protein p9Ka also contains two potential calcium-binding loops between residues 33 and 40 and residues 62 and 73 (Fig. 3). The C-terminal loop (residues 62–73) of p9Ka corresponds to an almost perfect EF-hand sequence, with five residues containing carboxylic acid derivatives in their side chains (aspartate 62, asparagine 64, aspartate 66, aspartate 70, glutamate 73 of the p9Ka amino acid sequence) in the exact positions of the loop region that are thought to be important in calcium binding by the vitamin D-dependent, intestinal calcium-binding protein (*72,73*). In p9Ka this potential binding site contains asparagine at position 67 instead of the smaller glycine residue. This latter residue is thought to play a role in maintaining the EF-hand structure in both S-100 protein and intestinal calcium-binding protein (*74*). However, this particular substitution may not prevent calcium binding by p9Ka, since the other, N-terminal loop of the intestinal calcium-binding protein also binds calcium, but does not contain this particular glycine residue (*72*).

It is not yet known whether one or both of the potential calcium-binding sites in p9Ka bind calcium under experimental or physiological conditions, or whether, as in the case of the S-100 protein, calcium binding is modulated by the presence of other cations, such as potassium and magnesium (*75*). The relative positions of the two potential binding sites in p9Ka are similar to those in a number of other small putative calcium-binding proteins (Fig. 4). The gene sequences corresponding to these proteins are not yet available. However, in the case of the gene for p9Ka the single intron interupts the hypothetical coding region in the linker between the two potential calcium-binding sites, thus separating the two functional domains (*62*).

In contrast, the genes of proteins with four calcium-binding sites, such as those of calmodulin (*76*) and Spec 1 protein (*77*), contain multiple introns that often interrupt the calcium-binding domains (Figs. 4 and 5). Whether all the small calcium-binding proteins have the same intron/exon arrangement is not known. It is interesting to speculate that the cytoskeletal rearrangement on differentiation of Rama 25 stem cells to the elongated myoepitheliallike cells (*53*) may be triggered, in part, by calcium-binding proteins acting directly on actin/myosin polymerization, or indirectly on tubulin polymerization, since artificial agents that disrupt microtubules trigger this change (*78*).

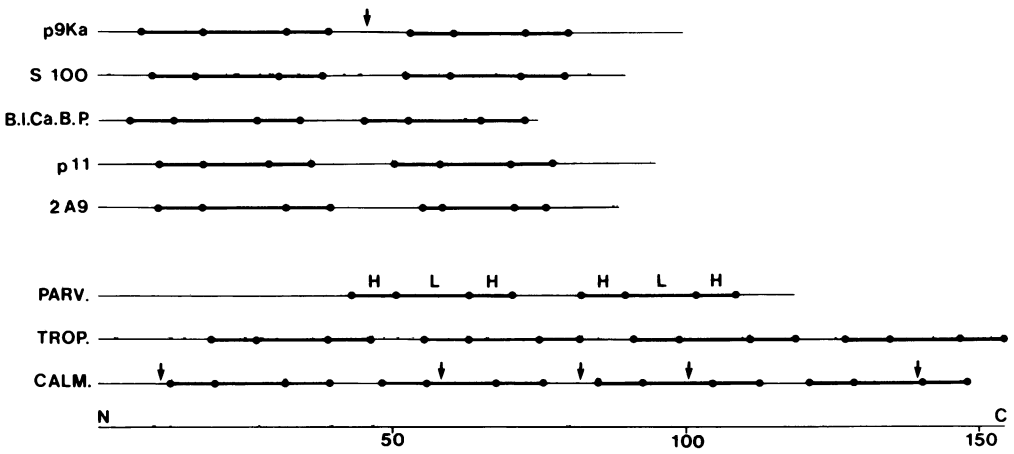

FIGURE 4. The location of potential calcium-binding regions in calcium-binding proteins. The positions of the helix (H), loop (L), helix (H) arrangement along the amino acid chains of some calcium-binding proteins are shown as heavy lines between the circles. The diagram has been constructed from the amino acid sequences of p9Ka, Barraclough et al. (*62*); S-100 protein (S 100), Isobe and Okuyama (*68*); bovine intestinal calcium-binding protein (B. I. Ca. B. P.), Fullmer and Wasserman (*70*); p11, Gerke and Weber (*88*); 2A9, Calabretta et al. (*89*); parvalbumin (PARV), Kretsinger (*90*); troponin C (TROP), Kretsinger (*90*); and calmodulin (CALM), Simmen et al. (*76*). The arrows denote the positions of introns in the corresponding gene sequences of p9Ka and calmodulin. Numbers are the number of residues from the N-terminus.

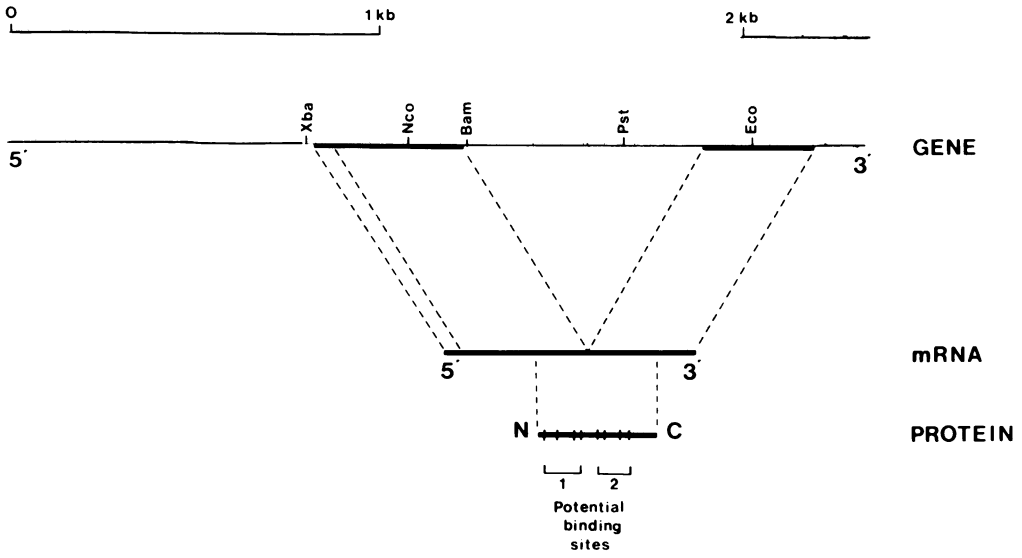

FIGURE 5. The transcription and processing of the rat p9Ka gene.

Changes Associated with Differentiation to Myoepitheliallike Cells *In Vitro* are Controlled at Different Levels of Genetic Expression

p9Ka, in addition to laminin, type IV collagen, and Thy-1, is a potential marker of myoepithelial cells *in vitro* and *in vivo*. In the elongated cell line Rama 29, Thy-1 antigen, Type IV collagen, laminin, and p9Ka are respectively 13-fold, 3.5-fold, 3.7-fold, and 16-fold more abundant than in the parental cuboidal, epithelial cells (*12,56,79*). Cloned cell lines intermediate in morphology between the cuboidal and elongated cells have been isolated and these form a series in the order Rama 25, Rama 25-I2, Rama 25-I1, Rama 25-I4, Rama 29, based on increasing elongated character, and, except for Rama 29, increasing frequency of conversion to the fully elongated cells (*56*). This same order is maintained for increases in myoepithelial characteristics and decreases in epithelial characteristics, including the marker proteins of the respective cell types. However, the individual changes do not occur to the same degree in the same intermediate cell line. Thus, the

majority of the increase in p9Ka occurs in the Rama 25-I1 cell line, whereas Thy-1 antigen increases predominantly in Rama 25-I1 and I4, and laminin increases almost equally in Rama 25-I2, I1, and I4 cells. Our results show that the production of these proteins is not synchronous, and therefore the regulation of their production is executed in an asynchronous manner as well.

This idea of asynchronous or stepwise regulation of the production of marker proteins for the myoepitheliallike cell is strengthened by a comparison of the relative levels of the mRNAs of the same marker proteins in the cuboidal, epithelial and elongated, myoepitheliallike cells. The levels of mRNA have been estimated by hybridizing poly(A)-containing RNA from the different cell lines to radioactive, cloned cDNAs corresponding to the mRNAs for Thy-1, p9Ka (*61*), laminin, and type IV collagen (Table 1). Thus, the 16-fold increase in the amount of Thy-1 in the elongated, myoepitheliallike cells can be completely accounted for by an equivalent increase in Thy-1 mRNA, as measured by hybridization to Thy-1 cDNA (*80*). In contrast, the 16-fold increase in p9Ka accumulation in the elongated, myoepitheliallike cells (*55*) is accompanied by only a 10-fold increase in p9Ka cytoplasmic mRNA (*61*). These results suggest that translational control may play a facilitating role in the increase in p9Ka. On the other hand, the 3.7-fold increase in laminin (*56*) and the 3.5-fold increase in type IV collagen (*79*) are accompanied by only a very small increase in their respective mRNAs (Table 1). The increase in type IV collagen seen in elongated, myoepitheliallike cells relative to the cuboidal, epithelial cells arises predominantly from a 5-fold decrease in the rate of intracellular breakdown of type IV collagen, estimated from the rate of release of hydroxyproline (*79*). The combination of these results strongly suggests that in the same cells, the steady-state levels of different marker proteins can arise by altering control at different molecular levels, including that at transcriptional, posttranscriptional processing or posttranslational steps. It has not yet been possible to determine whether the altered levels of Thy-1 mRNA arise from altered control at the initiation of transcription or at processing of the Thy-1 mRNA transcripts. These two levels of control can be distinguished by measuring the rate of completion of nascent RNA transcripts by isolated cell nuclei. However, in our experiments so far, Thy-1 RNA transcripts can be detected at only very low levels in nuclei from the elongated, myoepitheliallike cells and from the cuboidal epithelial cells. Under the same conditions, RNA transcripts corresponding to two other cellular mRNAs are readily detected (*80*). Thus, it is possible that putative factors necessary for the transcription of Thy-1 mRNA are lost during isolation of the nuclei from the elongated, cultured cells, but, at present, their identity is completely unknown.

In the case of p9Ka, preliminary evidence suggests that at least part of the increase in its accumulation in the myoepitheliallike cells *in vitro* arises from an increased rate of transcription of its mRNA when compared to the cuboidal epithelial cells (unpublished observation). In many genes, regions of DNA important in controlling the synthesis of their mRNA molecules are often located immediately adjacent to those sequences corresponding to the 5′ end of the mRNA. These regions, such as the TATA (*81*) and CAAT (*82*) concensus sequences are thought to be important in specifying the start site of transcription of the gene by RNA polymerase. However, no obvious TATA or CAAT promoter sequences are found close to the region corresponding to p9Ka mRNA. Genes that do not possess promoters that contain TATA or CAAT concensus sequences in either the coding or the noncoding strand of DNA have been identified recently (*83–87*). In at least two of these cases, the murine Thy-1,2 gene (*85,87*) and the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene (*84*) multiple initiation sites for the transcription of the mRNAs have been reported, similar to those found for the mRNA for p9Ka. The absence of TATA and CAAT promoter sequences is a characteristic of certain housekeeping genes (*84*) that possess promoter regions rich in GC nucleotide pairs, and short sequences, GGGCGG, homologous to part of the 21 nucleotide-pair repeat of the promoters for SV40 specific mRNAs (*84,86*). However, although the 5′ sequence flanking the gene for p9Ka does contain some G-rich regions, the above elements are absent. Thus, it is possible that the gene for p9Ka may contain a novel promoter that regulates its expression be-

Table 1. Relative levels of specific marker proteins and their mRNAs rat mammary cell lines.

Marker protein	Relative level of mRNA[a]		Reference
	Cuboidal, epithelial cell line (Rama 25)	Elongated, myoepitheliallike cell line (Rama 29)	
Thy-1 antigen			
Protein ratio	1	16.7	(*12*)
mRNA ratio 3 experiments	1	16.0 ± 2.1	(*80*)
p9Ka			
Protein ratio	1	16.3	(*55*)
mRNA ratio 4 experiments	1	10.3 ± 3.3	
Laminin			
Protein ratio	1	3.7	(*56*)
mRNA ratio 2 experiments	1	1.45 ± 0.2	(*80*)

[a]Poly(A)-containing RNA from cell lines was bound to nitrocellulose filters and the relative levels of specific mRNAs were found by quantitative hybridization to ^{32}P-labeled cloned cDNAs.

tween epithelial and myoepitheliallike cells. Similarly, the Thy-1 gene, which is also expressed in myoepitheliallike cells, but not in epithelial cells, also lacks CAAT and TATA concensus sequences and contains only a single region with strong homology to the GGGCGG sequence (*85*). A study of the functional promoters of the genes for these two proteins may lead to an understanding of some of the molecular control mechanisms of myoepithelial cell differentiation *in vitro* and *in vivo*.

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